FEBS Letters 589 (2015) 3309–3312

FEBS Letters
journal homepage: www.FEBSLetters.org

High pressure promotes alpha-synuclein aggregation in cultured neuronal cells

Urszula Golebiewska [a], Suzanne Scarlata [b,*]

[a] Dept. of Biological Sciences and Geology, Queensborough Community College, Bayside, NY 11364, USA
[b] Dept. of Chemistry and Biochemistry, Worcester Polytechnic Institute, Worcester, MA 01609 USA

ARTICLE INFO

Article history:
Received 13 July 2015
Revised 6 August 2015
Accepted 15 September 2015
Available online 3 October 2015

Edited by Jesus Avila

Keywords:
Parkinson's disease
Alpha synuclein
Phospholipase beta 1
Hydrostatic pressure
Aggregation

ABSTRACT

α-Synuclein is found in plaques associated with Parkinson's and other neurodegenerative diseases. Changes in α-synuclein oligomerization are thought to give rise to nucleation of neurodegenerative plaques. Here, we investigated the effect of hydrostatic pressure on the aggregation of α-synuclein in cultured neuronal cells. We found that hydrostatic pressure is associated with a transition from monomeric to higher order α-synuclein aggregates. We then tested whether this aggregation is associated with the loss of binding partners, such as phospholipase Cβ. We found that increased pressure reduces the level of PLCβ1 and the amount of α-synuclein/PLCβ1 complexes. These studies suggest that pressure promotes release of α-synuclein from protein partners promoting its oligomerization.

1. Introduction

Many neurodegenerative diseases, such as Parkinson's, Mutiple System Atrophy, Dementia with Lewy Bodies, and Amyotrophic Lateral Sclerosis, are caused by protein aggregates composed largely of the protein α-synuclein (AS). This group of neurodegenerative diseases, referred to as Lewy Body Diseases are found in patients that carry point mutations or multiple copies of the AS gene [1–9]. However, genetic mutations of AS account only for a small percentage of pathologies and two major environmental causes linked to sporadic LBD have been identified. One is traumatic brain injury (TBI) caused by acute force and pressure, followed by prolonged increased inter-cranial pressure. In neurons, this increased pressure affects the conformations and interactions of proteins which can lead to cell death [10–14].

AS is a small (140kDa) cytosolic protein that is abundant in the central nervous system, and conserved in vertebrates [1,15]. AS has been classified as a 'natively unstructured' protein [16]. However, under certain conditions, AS becomes helical and self-associates to a tetramer [17,18]. At high concentrations, AS forms fibrils which resemble the structures found in Lewy bodies. AS might also function as a 'prion' and internalize into neighboring neurons and further promote aggregation [19,20].

The cellular function of AS is unknown. Its high degree of conservation and abundance in neuronal tissue suggests that it plays some critical role. Studies of AS knock-out mice show a variety of neurological abnormalities that are difficult to interpret. Molecular-based studies suggest that AS can potentially bind many different partners that are primarily associated with lipid signaling and vesicle recycling [21]. Our lab has found that AS binds to the G-protein regulated enzyme phospholipase Cβ (PLCβ) with an affinity 10 times stronger than other reported protein partners [22]. Binding of AS to PLCβ alters its ability of hydrolyze signaling lipids and changes Ca^{2+} signaling in cells [22–26].

Because of the importance of AS in neurodegeneration, there have been many studies to delineate its cellular function, but no clear picture has emerged. Based on our work and the work of others, we proposed that AS does not have an intrinsic activity but rather modifies and optimizes cell function via interactions with other proteins [27]. Any stress that releases AS from its binding partners, such as oxidation or pressure, will result in AS aggregation and lead to cell death. In a recent study, we subjected cells to an oxidizing environment [26] that resulted in the loss of many cellular proteins including PLCβ while the cellular level of AS was not affected [26]. The decrease in PLCβ level promoted homo-oligomerization of released AS. Moreover, down-regulating PLCβ

Authors contributions: UG helped design the experiments, carried out the experimental work, analyzed data and helped prepare the manuscript. SS helped design the experiments, analyze results and prepare the manuscript.

* Corresponding author.
E-mail address: sfscarlata@wpi.edu (S. Scarlata).

http://dx.doi.org/10.1016/j.febslet.2015.09.019
0014-5793/

promoted AS aggregation while over-expressing PLCβ prevented AS aggregation.

It has been established that subjecting protein complexes to pressure, including AS fibrils, forces solvent into the void regions between the subunits resulting in disassembly [28,29]. Based on this observation, we propose that increased cranial pressure results in dissociation of AS from PLCβ, and other cell components that prevent its aggregation. The release of AS may then lead to oligomerization and ultimately, disruption of cell function (see [30]). In this study, we have tested this idea in cultured neuronal cells (PC12 and SK-N-SH) under hydrostatic pressure.

2. Materials and methods

2.1. Cell culture and transfection

SK-N-SH and PC12 cells were obtained from ATCC (HTB-11, CRL-1721). SK-N-SH cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin sulfate. PC12 cells were cultured in DMEM medium supplemented with 10% horse serum, 5% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin sulfate. Cells were kept at 37 °C with 5% CO_2. Plasmids were introduced at 80–90% confluency using Lypophectamine2000™ according to the manufacturer specifications. In general 1 μg of DNA was used for 10 μl of Lypophectamine2000™. Cells were transfected in serum free Opti-MEM medium and the medium was replaced with full growth medium after 5–7 h. Cells were imaged 48–72 h post transfection in phenol free Leibowitz15 (L15) medium.

2.2. Western blotting

Cells were lysed on ice for 20 min. Lysates were subjected to SDS–PAGE using 12% polyacrylamide gels, followed by the transfer to a nitrocellulose membrane. The membrane was then incubated for 1 h with 5% non-fat dry milk dissolved in Tris–buffered saline with 0.5% Tween (TBST) to block the non-specific binding. The primary antibody (mouse anti AS, rabbit anti PLCβ1), diluted according to the manufacturer's instructions, was added to the membrane and incubated for 1 h. This was followed by 3 washes of 15 min each in TBST. After the addition of horseradish peroxidase-conjugated secondary antibody for 1 h, the membrane was washed 3 times for 15 min each in TBST. The membrane was subjected to imaging after a chemiluminescence reaction (Pierce, Inc., Rockford, IL, USA).

2.3. In vivo single cell Föster resonance energy transfer (FRET)

FRET experiments were performed on Zeiss LSM 510 Meta/confocor2 apparatus (Jena, Germany) using synthesized emission method. We used a 40 × NA 1.2 C-Apochromat water immersion objective and following filter settings: GFP excitation: 488 nm line of argon ion laser and emission: 500–550 nm band pass filter, mCherry excitation: 543 nm line of HeNe laser and emission: 585–615 nm band pass filter, and FRET excitation: 488 nm and emission: 585–615 nm band pass filter. Bleed through from GFP fluorescence into the FRET channel and direct excitation of mCherry by the 488 nm laser line values were estimated from cells transfected with GFP-PLCβ1 and mCherry -AS separately and imaged under the appropriate filter sets. The negative control FRET value was determined from cells transfected with GFP-PLCβ1 and mCherry-vector. After background subtraction NFRET values were calculated for every pixel in the image according to formula:

$$\mathrm{NFRET} = \frac{I_{\mathrm{FRET}} - a \times I_{\mathrm{eGFP}} - b \times I_{\mathrm{mCherry}}}{\sqrt{I_{\mathrm{eGFP}} \times I_{\mathrm{mCherry}}}}$$

where a is the percentage of bleed-through of GFP through FRET filter set and b is the percentage of direct excitation of mCherry by 488 nm light [31].

2.4. Number and brightness (N&B)

PC12 cells were transfected with GFP-AS, subjected to hydrostatic pressure, plated in glass bottom chambers and allowed to recover for 24 h. Confocal images were collected on an Olympus Fluoview 1000 LSCM fitted with a 60× PlanApo (1.40 NA) oil immersion objective. The analog/digital hybrid detector (PMT) was used in photon counting mode with 1× gain and 0% offset. Images of cells were collected with resolutions of 66 nm/pixel. A region of interest (256 × 256 box or 128 × 128 box) was analyzed from an image of 320 × 320 pixels. The pixel dwell time was 4 μs/pixel and the pinhole diameter was 200 μm. GFP was excited with the 0.1% of 488-nm line of a 40 mW argon ion laser. In general 100 images of one cell were collected. Data analysis was done using the N&B analysis screen of the SimFCS program (www.lfd.uci.edu). The offset and the readout noise were determined from the histograms of dark counts performed after every 10 measurements (100 images collected with the laser turned off) [32].

2.5. Statistical analysis

For statistical analysis we used SigmaPlot13 (SPSS, INC., Chicago, IL). We compared the values of NFRET and N&B using Kruskal–Wallis One Way Analysis of Variance on Ranks, Dunn's Method, One Way Analysis of Variance, Tukey's Method, and t-test. We concluded the values are significantly different when $P < 0.05$.

3. Results

3.1. Overview of pressure studies

We first carried out preliminary studies to determine the amount of hydrostatic pressure that could be applied to cultured cells without a large loss in viable cells. We found that the neuronal cultured cell lines used in this study, human SK-N-SH and rat PC12, cells could withstand hydrostatic pressures of 1000 bars (comparable to the deepest point in the Pacific Ocean, and the highest pressure tested) for at least an hour with only a small fraction of the population lost. We note that pressure-treated cells attached identically to growth chambers as untreated cells and displayed normal morphology, growth and mitochondria.

In all studies, we subjected cells to either 500 or 1000 bars at room temperature (both gave identical results) by suspending the cells in medium, placing into a pressure chamber (see [33]) increasing the pressure over a 3–5 min period, incubating for a specific period of time, slowly releasing the pressure, plating the cells in chambers in growth medium, and allowing them to recover for 24 h. In all studies, control cells were treated identically except they were not pressurized.

3.2. Increased hydrostatic pressure promotes aggregation of intracellular endogenous AS

The level of AS aggregation of pressurized samples was analyzed by Western Blotting. The data for PC12 cells (Fig. 1A) show that pressure treatment results in higher molecular weight AS bands that correspond to a tetramer. Identical results were obtained for SK-N-SH cells and PC12 cells. We repeated the measurements multiple times with similar results.

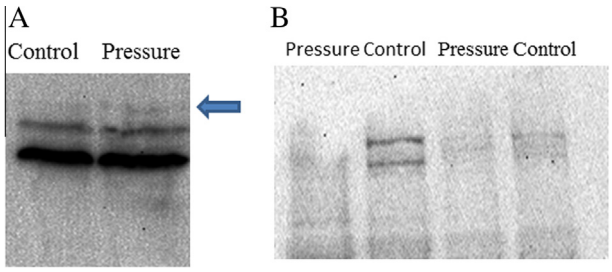

Fig. 1. Western blots of AS and PLCβ1 in PC12 cells treated with pressure. (A) PC12 cells were subjected to 1000 bars for 1 h, plated in growth medium and allowed to recover for 24 h. Control samples were kept at room temperature in identical chambers as the pressurized sample. Western blotting was carried out as described in Section 2. Pressure-treated samples showed a band at a molecular weight corresponding to a tetramer (marked with an arrow). (B) Pressure treated samples as in A were probed with rabbit anti PLCβ1. The blot shows two independent experiments. Two right columns are from the same lysates as in A. In both cases pressurized samples have significantly lower amount of PLCβ1.

3.3. Increased hydrostatic pressure lowers the endogenous level of PLCβ1

We hypothesized that the reason for increased aggregation of α-AS in pressure treated cells is the disappearance of binding partners. To test that possibility we analyzed pressure treated cell lysates for the presence of its binding partner PLCβ1. Pressure-treated PC12 cells were subjected to SDS–PAGE followed by western blotting. In Fig. 1B we show that pressure treated cells had reduced levels of PLCβ1 (the membrane was stripped and re-probed for AS to confirm its aggregation). The studies were repeated three times under similar conditions and at reduced pressure and exposure times and similar results we obtained.

3.4. Increased hydrostatic pressure reduces binding between AS and PLCβ1

To test whether increased hydrostatic pressure disrupts the interaction between AS and PLCβ1, we performed FRET measurements between GFP-labeled PLCβ1 and mCherry-labeled AS. PC12 cells were transfected for 24 h before pressurization. In Fig. 2, we show that the normalized FRET (NFRET) between GFP-PLCβ1 and mCherry-AS is significantly lower in the pressure-treated PC12 cells than in the untreated cells (pressure NFRET = 0.285 ± 0.006, $n = 70$; control NFRET = 0.325 ± 0.006, $n = 63$; $P < 0.001$). There was no change in signal between GFP-PLCβ1 and mCherry alone (pressure 0.250 ± 0.008, $n = 22$; control 0.261 ± 0.011, $n = 19$). These results show that pressure induces a loss in PLCβ1-AS association from ~14.0 to 6%.

3.5. Increased hydrostatic pressure increases aggregation of GFP-AS

To corroborate our Western Blot studies, we assessed the aggregation of AS in PC12 cells transfected with GFP-AS by N&B analysis. This method follows the number of photons associated with a diffusing species and can report on the oligomerization state. Cells were transfected with GFP-AS and half were subjected to pressure while the others used as controls. Next, the cells were plated in glass bottom dishes and N&B measurements were performed 24 h later. Fig. 3 shows that cells that were subjected to pressure had a higher value of Brightness as compared to untreated controls (brightness = 6.1 ± 0.3 * 10^4 counts/molecule/s with pressure ($n = 41$) and 4.6 ± 0.2 * 10^4 counts/molecule/s, control ($n = 40$), $P < 0.013$). These values can be compared to monomeric GFP controls in transfected PC12 cells (GFP with pressure: 4.1 ± 0.1 10^4 counts/molecule/s, $n = 28$ and GFP control: 4.3 ± 0.2 10^4 counts/molecule/s, $n = 38$). These results show that pressure induces AS aggregation.

4. Discussion

In this study, we show that subjecting neuronal cells to acute high pressure results in aggregation of AS. This result sharply contrasts the behavior seen using purified AS [28,29]. This aggregation was seen despite allowing the cells to recover for extended periods of time. In trying to understand the mechanism that underlies aggregation, we measured the loss of one of AS's binding partners, PLCβ1. Similar to the effects seen for cells subjected to oxidation, we find that the application of pressure reduces the association between PLCβ1 and AS most likely due to a loss in the cellular content of PLCβ1 as seen by western blotting. Dissociation of protein aggregates is commonly seen in pressure studies [33]. The increased AS aggregation is consistent with dissociation of AS-protein heterodimers and loss of AS binding partners.

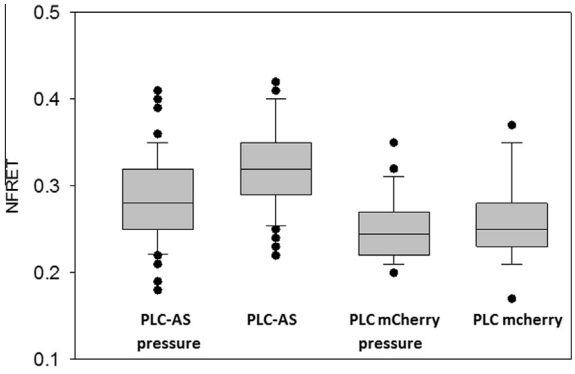

Fig. 2. Changes in PLCβ1-AS FRET in PC12 cells treated with pressure. Samples cells were transfected with GFP-PLCβ1 and mCherry-AS; Control cells were transfected with GFP-PLCβ1 and mCherry vector. 24 h post transfection cells were treated with 1000 bars for 1 h, plated in growth medium and imaged 24 h later. Distributions of NFRET values of pressure treated and control cells are shown in a box plot. The box represents 25th and 75th percentile, the line in the box represents median, and the whiskers represent 10th and 90th percentile. NFRET values of GFP-PLCβ1 and mCherry-AS for pressure treated samples and control are 0.285 ± 0.006, $n = 70$ and 0.325 ± 0.006, $n = 63$, respectively. The difference is statistically significant $P < 0.001$. The NFRET values of GFP-PLCβ1 and mCherry alone for pressure and control are 0.250 ± 0.008, $n = 22$ and 0.261 ± 0.011, $n = 19$.

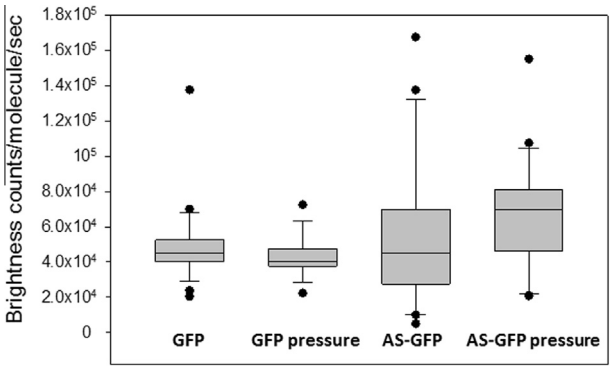

Fig. 3. Changes in GFP-AS in PC12 cells treated with pressure. PC12 cells were transfected with GFP-AS or GFP vector, 24 h post transfection cells were treated with 1000 bars for 1 h, plated in growth medium and imaged 24 h later. The box plot (see above) represents distribution of the brightness per molecule per second. Brightness of the pressure and control samples is 6.1 ± 0.3 * 10^4 counts/molecule/s, $n = 41$ and 4.6 ± 0.2 10^4 counts/molecule/s, $n = 40$ respectively. The difference is statistically significant $P < 0.013$. Brightness of GFP controls in transfected PC12 cells with pressure: 4.1 ± 0.1 10^4 counts/molecule/s, $n = 28$ and control: 4.3 ± 0.2 10^4 counts/molecule/s, $n = 38$.

The amount of transient pressure that occurs upon head injury is quite variable but may very high in some cases. However, intracranial pressures are far lower than the pressure used here, but have a longer duration. Because cultured cells will not survive well in the pressure chamber for extended periods, we subjected cells to very high pressure for short period of time but stress that it is unclear how these pressures compare to those experienced during injury. We note that the pressures used here are experienced by biological organisms under extreme conditions.

Acknowledgements

We acknowledge Osama Garwain, Siddartha Yerramilli, and Siyuan Xue for technical assistance.

This work was supported by NIH GM116178 to SS and PSC-CUNY 45 to UG.

References

[1] George, J.M. (2002) The synucleins. Genome Biol. 3, 3002.1–3002.6.
[2] Parsian, A., Racette, B., Zhang, Z.H., Chakraverty, S., Rundle, M., Goate, A. and Perlmutter, J.S. (1998) Mutation, sequence analysis, and association studies of alpha-synuclein in Parkinson's disease. Neurology 51, 1757–1759.
[3] Duda, J.E., Lee, V.M. and Trojanowski, J.Q. (2000) Neuropathology of synuclein aggregates. J. Neurosci. Res. 61, 121–127.
[4] Lundvig, D., Lindersson, E. and Jensen, P.H. (2005) Pathogenic effects of α-synuclein aggregation. Mol. Brain Res. 134, 3–17.
[5] Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E.S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W.G., Lazzarini, A.M., Duvoisin, R.C., Di Iorio, G., Golbe, L.I. and Nussbaum, R.L. (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 276, 2045–2047.
[6] Zarranz, J.J., Alegre, J., Gomez-Esteban, J.C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atares, B., Llorens, V., Gomez Tortosa, E., del Ser, T., Munoz, D.G. and de Yebenes, J.G. (2004) The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Ann. Neurol. 55, 164–173.
[7] Conway, K.A., Harper, J.D. and Lansbury, P.T. (1998) Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease. Nat. Med. 4, 1318–1320.
[8] Narhi, L., Wood, S.J., Steavenson, S., Jiang, Y., Wu, G.M., Anafi, D., Kaufman, S.A., Martin, F., Sitney, K., Denis, P., Louis, J.C., Wypych, J., Biere, A.L. and Citron, M. (1999) Both familial Parkinson's disease mutations accelerate alpha-synuclein aggregation. J. Biol. Chem. 274, 9843–9846.
[9] Pandey, N., Schmidt, R.E. and Galvin, J.E. (2006) The alpha-synuclein mutation E46K promotes aggregation in cultured cells. Exp. Neurol. 197, 515–520.
[10] Nayernouri, T. (1985) Posttraumatic parkinsonism. Surg. Neurol. 24, 263–264.
[11] Factor, S.A. and Weiner, W.J. (1991) Prior history of head trauma in Parkinson's disease. Mov. Disord. 6, 225–229.
[12] Stern, M.B. (1991) Head trauma as a risk factor for Parkinson's disease. Mov. Disord. 6, 95–97.
[13] Lees, A.J. (1997) Trauma and Parkinson disease. Revue Neurol. 153, 541–546.
[14] Goldman, S.M., Tanner, C.M., Oakes, D., Bhudhikanok, G.S., Gupta, A. and Langston, J.W. (2006) Head injury and Parkinson's disease risk in twins. Ann. Neurol. 60, 65–72.
[15] Clayton, D.F. and George, J.M. (1998) The synucleins: a family of proteins involved in synaptic function, plasticity, neurodegeneration and disease. Trends Neurosci. 21, 249–254.
[16] Weinreb, P.H., Zhen, W., Poon, A.W., Conway, K.A. and Lansbury Jr., P.T. (1996) NACP, a protein implicated in Alzheimer's disease and learning, is natively unfolded. Biochemistry 35, 13709–13715.
[17] Bartels, T., Choi, J.G. and Selkoe, D.J. (2011) [agr]-Synuclein occurs physiologically as a helically folded tetramer that resists aggregation. Nature 477, 107–110.
[18] Wang, W., Perovic, I., Chittuluru, J., Kaganovich, A., Nguyen, L.T., Liao, J., Auclair, J.R., Johnson, D., Landeru, A., Simorellis, A.K., Ju, S., Cookson, M.R., Asturias, F.J., Agar, J.N., Webb, B.N., Kang, C., Ringe, D., Petsko, G.A., Pochapsky, T.C. and Hoang, Q.Q. (2011) A soluble alpha-synuclein construct forms a dynamic tetramer. Proc. Natl. Acad. Sci. U.S.A. 108, 17797–17802.
[19] Dunning, C.J., George, S. and Brundin, P. (2013) What's to like about the prion-like hypothesis for the spreading of aggregated alpha-synuclein in Parkinson disease? Prion 7, 92–97.
[20] George, S., Rey, N.L., Reichenbach, N., Steiner, J.A. and Brundin, P. (2013) Alpha-synuclein: the long distance runner. Brain Pathol. 23, 350–357.
[21] Jin, J., Li, G.J., Davis, J., Zhu, D., Wang, Y., Pan, C. and Zhang, J. (2007) Identification of novel proteins associated with both alpha-synuclein and DJ-1. Mol. Cell. Proteomics 6, 845–859.
[22] Narayanan, V., Guo, Y. and Scarlata, S. (2005) Fluorescence studies suggest a role for α-synuclein in the phosphatdiylinositol lipid signaling pathway. Biochemistry 44, 462–470.
[23] Golebiewska, U.P., Zurawsky, C. and Scarlata, S. (2012) Gamma synuclein forms tertramers that can be disrupted by phospholipase C. Biophys. J. 102.
[24] Golebiewska, U., Zurawsky, C. and Scarlata, S. (2014) Defining the oligomerization state of gamma-synuclein in solution and in cells. Biochemistry 53, 293–299.
[25] Guo, Y., Rosati, B. and Scarlata, S. (2012) α-Synuclein increases the cellular level of phospholipase Cβ1. Cell. Signal. 24, 1109–1114.
[26] Guo, Y. and Scarlata, S. (2013) A loss in cellular protein partners promotes α-synuclein aggregation in cells resulting from oxidative stress. Biochemistry 52, 3913–3920.
[27] Scarlata, S. and Golebiewska, U. (2014) Linking alpha-synuclein properties with oxidation: a hypothesis on a mechanism underling cellular aggregation. J. Bioenergy Biomembr. 46, 93–98.
[28] Cordeiro, Y., Foguel, D. and Silva, J.L. (2013) Pressure-temperature folding landscape in proteins involved in neurodegenerative diseases and cancer. Biophys. Chem. 183, 9–18.
[29] Foguel, D., Suarez, M.C., Ferrao-Gonzales, A.D., Porto, T.C., Palmieri, L., Einsiedler, C.M., Andrade, L.R., Lashuel, H.A., Lansbury, P.T., Kelly, J.W. and Silva, J.L. (2003) Dissociation of amyloid fibrils of alpha-synuclein and transthyretin by pressure reveals their reversible nature and the formation of water-excluded cavities. Proc. Natl. Acad. Sci. U.S.A. 100, 9831–9836.
[30] Scarlata, S. and Golebiewska, U. (2014) Linking alpha-synuclein properties with oxidation: a hypothesis on a mechanism underling cellular aggregation. J. Bioenergy Biomembr. 46, 93–98.
[31] Xia, Z. and Liu, Y. (2001) Reliable and global measurement of fluorescence resonance energy transfer using fluorescence microscopes. Biophys. J. 81, 2395–2402.
[32] Dalal, R.B., Digman, M.A., Horwitz, A.F., Vetri, V. and Gratton, E. (2008) Determination of particle number and brightness using a laser scanning confocal microscope operating in the analog mode. Microsc. Res. Tech. 71, 69–81.
[33] Royer, C.A., Weber, G., Daly, T.J. and Matthews, K.S. (1986) Dissociation of the lactose repressor protein tetramer using high hydrostatic pressure. Biochemistry 25, 8308–8315.